Association with outcomes and response to treatment of trimethylamine N-oxide in heart failure (from BIOSTAT-CHF)

Suzuki, Toru ; Yazaki, Yoshiyuki ; Voors, Adriaan A.; Jones, Donald J. l.; Chan, Daniel C. S.; Anker, Stefan D.

Published in:
European Journal of Heart Failure

DOI:
10.1002/ejhf.1338

Publication date:
2019

Document Version
Peer reviewed version

This is the accepted manuscript version of Suzuki, Toru et al."Association with outcomes and response to treatment of trimethylamine N-oxide in heart failure (from BIOSTAT-CHF)". European Journal of Heart Failure. 2018. Final version available via DOI: 10.1002/ejhf.1338

Association with outcomes and response to treatment of trimethylamine N-oxide in heart failure (from BIOSTAT-CHF)

Short Title: TMAO and heart failure treatment in BIOSTAT-CHF

Toru Suzuki[*1], Yoshiyuki Yazaki[*1], Adriaan A Voors[2], Donald JL Jones[1,3], Daniel CS Chan[1], Stefan D Anker[4,5], John G Cleland[6], Kenneth Dickstein[7,8], Gerasimos Filippatos[9], Hans L Hillege[2], Chim C Lang[10], Peter Ponikowski[11], Nilesh J Samani[1], Dirk J Van Veldhuisen[2], Faiez Zannad[12], Aeiko H Zwinderman[13], Marco Metra[14], Leong L Ng[1]

* These authors contributed equally

Affiliations:

[1] Department of Cardiovascular Sciences, University of Leicester, Leicester, NIHR Leicester Biomedical Research Centre, Leicester, United Kingdom

[2] University of Groningen, University Medical Center Groningen, Department of Cardiology, Groningen, The Netherlands

[3] Department of Cancer Studies, University of Leicester, Leicester Royal Infirmary, Leicester, United Kingdom

[4] Division of Cardiology and Metabolism – Heart Failure, Cachexia & Sarcopenia, Department of Cardiology (CVK); and Berlin-Brandenburg Center for Regenerative Therapies (BCRT); Deutsches Zentrum für Herz-Kreislauf-Forschung (DZHK) Berlin; Charité Universitätsmedizin Berlin, Germany

[5] Department of Cardiology and Pneumology, University Medical Center Göttingen (UMG), Göttingen, Germany

[6] National Heart & Lung Institute, Royal Brompton and Harefield Hospitals, Imperial College, London, UK

[7] University of Bergen, Bergen, Norway

[8] Stavanger University Hospital, Stavanger, Norway

[9] National and Kapodistrian University of Athens, School of Medicine, Department of Cardiology, Heart Failure Unit, Athens University Hospital Attikon, Athens, Greece

[10] School of Medicine Centre for Cardiovascular and Lung Biology, Division of Medical Sciences, University of Dundee, Ninewells Hospital & Medical School, Dundee, United Kingdom

[11] Department of Heart Diseases, Wroclaw Medical University, Poland and Cardiology Department, Military Hospital, Wroclaw, Poland

[12] Inserm CIC 1433, Université de Lorraine, CHU de Nancy, Nancy, France

[13] Department of Epidemiology, Biostatistics and Bioinformatics, Academic Medical Centre, Amsterdam, the Netherlands

[14] Institute of Cardiology, Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, Italy

Address for Correspondence:

Toru Suzuki or Leong Ng

Department of Cardiovascular Sciences and NIHR Leicester Cardiovascular Biomedical Research Centre

University of Leicester, Glenfield Hospital, Leicester, LE3 9QP, United Kingdom

Email: ts263@le.ac.uk or lln1@le.ac.uk. Tel: (0044) 116 204 4741

Word Count: 5,248

ABSTRACT

AIMS Association of elevated circulating levels of trimethylamine N-oxide (TMAO) with adverse outcomes in patients with heart failure (HF) has been described. However, response of TMAO levels to treatment and medications has not been investigated. Therefore, we investigated whether TMAO levels are responsive to guideline-recommended treatment and medications, and further reflect changes in outcomes.

METHODS AND RESULTS TMAO levels were investigated in the systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF), which addressed response to guideline-recommended pharmacological treatment. TMAO levels in 2,234 patients with new-onset or progressively worsening HF showed strong associations with adverse events (mortality and /or rehospitalisation) at 1,2 and 3 years (HR 1.37–1.51, $p \leq 0.019$). Analysis of 972 patients with plasma available at both enrolment and follow-up visit showed reductions of B-type natriuretic peptide levels with guideline-based treatment ($p<0.001$), but not for TMAO levels. Moreover, patients with higher TMAO levels than median before and after treatment showed increased association with adverse outcomes (HR 2.21, 95% CI: 1.43-3.43, $p<0.001$) compared to patients with lower than median levels either before or after treatment (HR 1.13, 95% CI: 0.63-2.04, $p=0.684$ and HR 1.14, 95% CI: 0.64-2.03, $p=0.662$, respectively).

CONCLUSION TMAO levels were associated with adverse outcomes (mortality and/or rehospitalisation) in BIOSTAT-CHF, and did not respond to guideline-based pharmaceutical treatment in contrast to BNP levels which did as expected. Lower TMAO levels regardless of treatment were associated with favorable outcome.

Key Words: Heart failure, gut microbiome, biomarker, metabolite, outcome study

INTRODUCTION

The pathophysiology underlying heart failure (HF) is complex with multifaceted contributions of mechanical and neurohormonal factors and their collective effects on the heart.[1] Recently, the contribution of the gut microbiome to heart failure has been a topic of interest with the identification of the phosphatidylcholine metabolite, trimethylamine N-oxide (TMAO), to be a gut microbiota-derived molecule whose circulating levels when elevated are strongly associated with adverse outcomes for both acute[2] and chronic HF[3-7] as determined by cross-sectional studies. TMAO production involves gut microbiome-mediated processing of carnitine and/or choline (e.g. red meat and egg yolk as dietary sources) to the precursor trimethylamine (TMA) which is then converted to TMAO by flavin-containing monooxygenase 3 (FMO3) in the liver.[8,9] TMAO cannot be produced effectively if the gut microbiome is absent (e.g. antibiotics) or the diet is vegan/vegetarian-based.[10] Whether TMAO levels are responsive to medication and treatment, and further reflects changes in outcomes remains unknown. We hypothesized that TMAO levels would not respond to guideline directed medical therapy as conventional medications for HF do not target the gut microbiome.

The systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) was a European multicentre, prospective, observational project involving 69 centres in 11 countries[11], designed to implement current European guidelines of HF treatment[12] and to characterise biological pathways related to drug responsiveness to guideline-recommended pharmacological treatment for HF.

The present study investigated the association of circulating TMAO levels to outcomes in this contemporary European HF cohort with a particular focus on association of serial TMAO levels with treatment and outcomes.

METHODS

Study Population

BIOSTAT-CHF has been described in full elsewhere.[11] In brief, the BIOSTAT-CHF cohort enrolled 2516 patients in total between 2010-2014 with progressive worsening or new-onset symptoms of HF, confirmed by either a left ventricular ejection fraction (EF) of ≤40% or plasma concentrations of B-type natriuretic peptide (BNP) and/or N-terminal pro-B-type natriuretic peptide (NT-proBNP) >400pg/ml or >2000pg/ml, respectively. The main aim of the project was to establish the effects of and response to initiation and up-titration of guideline-directed medical therapy, therefore all patients underwent treatment with furosemide ≥40mg/day or equivalent and received ≤50% of target doses of angiotensin-converting-enzyme inhibitors or angiotensin II receptors (ACEi/ARBs) and beta-blockers at time of study entry. Each patient consented (written and informed) to have blood samples taken and outcomes surveyed. The study complied with the Declaration of Helsinki and was approved by the local ethics committee.

Sample Collection

Blood samples were collected at the initial enrolment visit (V1) in all patients and at a follow-up visit (V2) at approximately nine months in possible patients.

Biomarker Measurements

Plasma was aliquoted and stored at -80 ºC until analysis. At the time of analysis, samples were defrosted at room temperature and analysed immediately. TMAO levels were measured in plasma samples using stable isotope dilution [d9-(trimethyl)-labelled TMAO] followed by ultra-performance liquid chromatography-tandem mass spectrometry on a Quattro Premier XE triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), using conditions described previously with a coefficient of variation of 5.7% for measurements throughout the study. [2,13] All

further clinical biomarkers were measured either at local hospital site or within the BIOSTAT-CHF central laboratory.[11] BNP was measured using Luminex multiplexed bead-based immunoassays at Alere (San Diego, California).

Endpoints

The primary outcome was all-cause mortality (mortality), with a secondary outcome measure as the composite event of mortality combined with rehospitalisation due to HF (mortality/HF). End points were obtained as previously reported for the BIOSTAT-CHF protocol.[11]

Statistical Analyses

Investigations were performed on a non-imputed BIOSTAT-CHF database and modifications were made where sufficient data were not available. For patients that completed a second visit, changes in demographics from initial enrolment visit (V1) to the follow-up visit (V2) were compared using Wilcoxon matched-pair signed-rank test for continuous variables and the McNemar test for categorical variables. Cumulative incidences of events were investigated using Kaplan-Meier survival curves for tertiles of TMAO at baseline and compared using the Mantel-Cox log rank test. Cox proportional hazards regression analyses were performed to investigate association of plasma TMAO levels with outcomes in both a univariable and multivariable manner.

Multivariable adjustments were made using the previously defined BIOSTAT-CHF compact and extended risk models.[14]

To investigate the implications of serial changes in plasma TMAO levels with treatment on outcomes, patients with data available for both V1 and V2 were further classified according to respective TMAO levels at each time point. In total, four groups were created and classified by low or high TMAO levels (according to the median) for each visit. Cox proportional hazards

regression analysis was performed to investigate the change in associations with outcomes across groupings, with low V1 and low V2 (L/L group) deemed as the reference value. Similar analysis was done with natriuretic peptide levels. Decision tree analysis was performed using χ^2 automatic interaction detection (CHAID) and resultant groupings tested for association with outcomes using Cox proportional hazards regression and Kaplan-Meier survival curves.

Statistical analyses were performed using IBM SPSS Statistics (v24, IBM, Armonk, NY, USA). A P-value of <0.05 was considered statistically significant.

RESULTS

Study Population

Plasma TMAO levels were determined in 2234 patients (88.8%) with available baseline plasma samples of the 2516 patients that were included in BIOSTAT-CHF.

Patient demographics are shown in **Table 1.** The cohort consisted of 2234 patients with an age of 70 [61–78] years (median [interquartile range]) with 74% being male and 32% with history of previous HF hospitalisation. Patients were predominantly New York Heart Association (NYHA) functional classification of HF classes II and III (combined 86%), and presented with HF with reduced ejection fraction (EF) in 81%.

Patient characteristics according to TMAO median and tertiles are shown in **Supplementary Table S1**. Patients with higher TMAO levels at baseline had more comorbidities, advanced heart failure and worsened renal function as well as less use of ACEi/ARB and mineralocorticoid receptor antagonist (MRA).

972 out of the 2234 patients were analysed at follow up for TMAO levels (320 patients died before 9 months follow-up resulting in 1914 patients remaining of whom samples were available in 972 patients).

This subset of 972 patients had undergone guideline-based treatment for HF and showed improvement in clinical variables (**Table 1**) as shown by reduction of congestion and peripheral oedema (-38% and -27%, $p<0.001$), reduction in BNP levels (from 206 [86-408] pg/mL to 132 [51-327] pg/mL, $p<0.001$), reduction in NYHA class (e.g. 48% to 24% for NYHA class III) and improvement of EF (30% [25-35] to 35% [28-42], $p<0.001$). Use of beta-blocker, ACEi/ARBs and MRA were increased (85% to 93%, 74% to 89%, and 52% to 59% respectively, all $p<0.001$), and use of diuretics was reduced (100% to 94%, $p<0.001$) between V1 and V2.

Differences in characteristics between patients with and without TMAO measurements at V1 and V2 are shown in **Supplementary Table S2**. More males were in the group with TMAO

measured as compared to those not measured at V1, and those not measured at V2 showed lower use of beta-blockers and ACEi/ARBs, and lower eGFR levels.

Association of Circulating TMAO Levels with Adverse Outcomes

During the 3-year observation period, there were 591 mortality events (26.5%) and 909 composite events of mortality/HF (40.7%). To understand the association of baseline plasma TMAO levels and outcomes, Kaplan-Meier survival analyses were performed across TMAO tertiles (tertile1=<4.2 μmol/L; tertile2=4.2-8.4 μmol/L; tertile3=>8.4 μmol/L). Patients with elevated TMAO levels were associated with more deaths and composite events during the observation period, with a graded increase in the cumulative incidence of events with increasing TMAO levels ($p<0.001$, **Figure 1**).

To investigate the association of TMAO levels with outcomes, log TMAO levels were added to the previously described BIOSTAT-CHF compact and extended risk models for mortality and mortality/HF at 1, 2 and 3 years (11) (**Table 2**). The BIOSTAT-CHF compact risk model for mortality included age, haemoglobin, blood urea, log BNP and use of beta-blocker at baseline. BNP levels were substituted for NT-proBNP due to insufficient availability of data (data available in only 47% patients for NT-proBNP). When adjusted for this model, TMAO levels were associated with mortality and mortality/HF at 1, 2 and 3 years (HR 1.37-1.51, $p\leq0.019$). The BIOSTAT-CHF extended risk model for mortality included ischaemic aetiology, previous chronic obstructive pulmonary disease, diastolic blood pressure (BP) and sodium in addition to variables previously described for the compact model of mortality. When adjustment using this model was performed, TMAO remained an independent predictor of outcome for mortality at all time points (HR 1.40-1.44, $p\leq0.030$) (see **Table 2** for detailed statistical results). The BIOSTAT-CHF compact risk model for mortality/HF included age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, log BNP, haemoglobin, sodium and use of beta-blocker at baseline. High-density lipoprotein data were excluded due to availability in only 1036 patients

(46%). TMAO levels were associated with mortality/HF at all time points ($p \leq 0.019$). Finally, the BIOSTAT-CHF extended risk model for mortality/HF included current smoking, previous chronic obstructive pulmonary disease and estimated glomerular filtration rate (eGFR) in addition to the variables of the compact model. Presence of raised jugular vein dilatation was excluded due to low availability (69%). However, in this model, TMAO was not able to independently predict mortality/HF at any time point ($p \geq 0.054$). Therefore, TMAO levels showed added value to risk models for association with mortality outcome and mortality/HF at 1, 2 and 3 years, except for the extended model for mortality/HF. Associations of TMAO on outcome were not affected when adjusted for body mass index (BMI) on mortality and mortality/HF, except for the extended model of mortality/HF (**Supplementary Table S3**).

When TMAO was added to the BIOSTAT-CHF compact and extended models for mortality at 2 years, there were only marginal gains in C-statistics (compact model with vs without TMAO = 0.710 vs 0.705, p=0.138; extended model with vs without TMAO = 0.728 vs 0.723, p=0.073) (**Supplementary Table S4**). However, net reclassification index (NRI) [16.8 (95%CI: 6.6-27.1), p=0.001, 13.4 (3.0-23.9), p=0.012, respectively] and integrated discrimination improvement (IDI) analyses [0.5 (0.2-0.9), p=0.004, 0.5 (0.2-0.8), p=0.004, respectively] demonstrated the added value of TMAO (**Supplementary Table S4**). To note, reclassification analysis is known to offer greater sensitivity in highlighting improvement for the inclusion of an additional variable in comparison to C-statistic analysis[15].

In patients with EF<40% (n=1619), TMAO levels showed added value to BIOSTAT-CHF risk models for association with mortality outcome and mortality/HF at 1, 2 and 3 years, except for the extended model for mortality/HF at 1 year ($HR \geq 1.27$, $p \leq 0.041$, see **Supplementary Table S5** for detailed statistical results). However, this association was not seen in patients with EF≥40%. BIOSTAT-CHF did not assess echocardiographic parameters beyond EF as needed for classification of HF phenotype (e.g. HFrEF, HFpEF), therefore effects of HF phenotype could not be further investigated.

Association of Serial Changes in TMAO Levels with Adverse Outcomes

To understand the associations of subsequent adverse outcomes with serial changes in circulating TMAO levels following treatment, TMAO levels at enrolment (V1) and at the follow-up (V2) time point at nine months after treatment were split into high and low level groups (in relation to median values; V1 median, 5.7 μmol/L; V2 median, 6.6 μmol/L). The groups were comprised of low V1 and low V2 (L/L, n=315), low V1 and high V2 (L/H, n=171), high V1 and low V2 (H/L, n=171), and high V1 and high V2 (H/H, n=315) levels. Analysis showed that patients with low TMAO levels at V1 with high levels at V2 (L/H) and those who showed high levels at V1 with low levels at V2 (H/L) did not show an increased association with mortality at 2 years following the follow-up visit as compared to the reference L/L group [HR (95% confidence intervals); 1.13 (0.63-2.04) and 1.14 (0.64-2.03) for L/H and H/L, respectively]. However, patients who showed high TMAO levels at both time points (H/H) showed increased association with mortality at 2 years after the follow-up visit [HR 2.21 (1.43-3.43), p<0.001] (**Figure 2A**). Thus, increased association with mortality was seen in patients with sustained increases in TMAO levels at both enrolment and follow-up, or conversely, increased association was not seen in patients that exhibited low TMAO levels at either visit.

Similar analysis was done with natriuretic peptide levels (**Figure 2C**). Patients were divided into four groups by median BNP levels for V1 and V2 (V1 median, 206 pg/mL; V2 median, 132 pg/mL). This produced groups of 297, 148, 148, and 297 patients for L/L, L/H, H/L, and H/H groups, respectively. Patients who showed low BNP levels following an initially high level (H/L) showed a similar association with mortality (HR 1.17 (0.55-2.48), p=0.679) as compared to the L/L group. Patients with high levels of BNP at V2 (L/H and H/H) showed increased association with mortality at 2 years [L/H HR 2.70 (1.45-4.99), p=0.002; H/H HR 3.56 (2.11-6.00), p<0.001]. Thus, natriuretic peptide showed a different trend where low natriuretic peptide levels after treatment at follow-up were associated with better outcomes, and high levels at follow-up were associated with worse outcomes regardless of initial levels.

To note, these associations were also seen when adjusted for renal function (**Figures 2B, D**). Changes in TMAO were associated with mortality even when adjusted for potential confounders such as BMI, systolic BP, EF and renal function (eGFR) (**Supplementary Table S6**).

These associations were also seen when patients were divided into tertiles (high/mid/low TMAO levels) at V1 and V2 instead of using median levels to split into high/low groups (**Supplementary Figure S1**). Tertile analysis showed that high to high, mid to high, and high to mid level changes showed association with adverse outcome consistent with those of sustained high levels from V1 to V2 using median analysis thus confirming that sustained high levels of TMAO are associated with adverse outcome.

Analysis was also done in patients with EF<40% (n=713) (**supplementary Table S7**). Association with mortality was seen in patients with sustained increases in TMAO levels at both V1 and V2 even after adjustment for potential confounders (BMI, systolic BP, EF and eGFR).

Decision Tree Analysis

Decision tree analysis was done to investigate TMAO levels at V1 and V2 as risk stratification biomarkers for mortality at 2 years following V2 (**Figure 3A**). This showed that stratifying patients by median TMAO levels at V1 and V2 generated three groups of risk that confirmed associations reported by the previous Cox proportional hazards regression analysis. Those with TMAO levels below the median at V1 (group A) showed a similar level of risk to those who initially presented with high levels that subsequently showed low levels (group B); relative risks (RR) of groups A and B were 10.7% and 12.5%, respectively. Conversely, those who presented with and maintained high levels of circulating TMAO (group C) showed an increased level of risk (RR of 26.0%; and HR 2.10 (1.44-3.06), p<0.001 compared to group A). Kaplan-Meier survival analysis confirmed that group C had an increased number of cumulative events compared to groups A and B (log-rank p<0.001, **Figure 3A** inset).

Similar analysis was also done on natriuretic peptide levels (**Figure 3B**). Decision tree analysis showed that a high level of BNP at V2 (>132 pg/mL) was associated with worse prognosis irrespective of measurement levels at the initial visit. Patients who had low BNP levels at V2 (groups A and C) showed the lowest level of risk (RR of 6.5% and 8.0% for groups A and C, respectively), and those with high levels at V2 (groups B and D) showing increased levels of risk (group RR of 18.4% and 26.7% for groups B and D, respectively). Kaplan-Meier survival analysis confirmed similar levels of cumulative events for groups A and C, and increase in events for groups B and D (log-rank $p<0.001$, **Figure 3B** inset). These results showed effects of TMAO on predictive values by cut-off levels at both V1 and V2, and therefore highlighted the importance of serial measurements.

Response of Circulating TMAO Levels to Treatment and Association with Outcomes

Response of TMAO levels to treatment was also investigated (**Table 3**) as BIOSTAT-CHF had also recorded dosage titrations. We hypothesized that TMAO levels would not respond to guideline directed medical therapy as conventional medications for treatment of HF do not target the gut microbiome in contrast to BNP, the comparator and reference, which is known to respond to treatment with lower levels[16]. For patients using ACEi/ARBs, regardless of whether they had achieved optimal titration to recommended dosage or not, TMAO levels showed increases by V2. Beta-blocker use showed increases in TMAO when less than 50% optimal dosage was used but not when higher levels of optimization were achieved. When combined for these drugs, less than 50% optimal titration showed increase in TMAO levels but not when higher levels of titration were achieved. In contrast, BNP showed reduced levels when greater than 50% optimal titration of either or both ACEi/ARBs and/or beta-blocker were used, but not when less than 50% optimal titration was achieved. TMAO levels were not responsive to optimized current guideline-based HF treatment in contrast to BNP levels.

In patients with EF<40%, TMAO levels showed increases regardless of optimization of ACEi/ARBs, and those on beta-blockers did not show significant difference in patients with greater than 50% optimal titration but showed significant increase in those with less than 50% optimal titration (**Supplementary Table S8**).

Titration of ACEi/ARBs, beta-blockers or both did not show interaction with TMAO levels on effects on mortality (ACEi/ARBs, beta-blockers or both; $P_{interaction}$ =1.00, $P_{interaction}$ =0.242 and $P_{interaction}$=0.442, respectively).

DISCUSSION

The present analysis of BIOSTAT-CHF validates that initial TMAO levels are associated with adverse outcomes (mortality and mortality/HF). In contrast to natriuretic peptides which responded to treatment as expected, TMAO did not respond to guideline medical treatment. Patients with sustained higher levels of TMAO before and after treatment were associated with worse outcomes, and patients with lower levels either before or after treatment did not show additional risk.

Comparison to Previous Studies

The present study investigated the role of the gut microbiome-derived metabolite biomarker of HF, TMAO, in the BIOSTAT-CHF cohort. As compared to previous acute and chronic HF patient cohorts in which TMAO levels have been investigated[2-7], the BIOSTAT-CHF cohort included over 2000 patients with progressive worsening or new-onset symptoms of HF. In the present cohort, increased TMAO levels were independently associated with adverse outcomes of mortality and a composite endpoint of mortality and hospitalization due to HF. Taken together with past observations in acute and chronic HF cohorts[2-7], elevated TMAO levels are consistently associated with adverse outcomes in patients with HF (mortality and/or HF rehospitalisation).

Novel Findings from the Present Study

One of the main aims of BIOSTAT-CHF was to establish the effects of response to initiation and up-titration of guideline-directed medical therapy in HF patients.[11] Drug therapy at enrolment, changes in the use of medications and up-titration to doses were recorded allowing for analysis of TMAO levels over time in response to and association with outcomes after treatment which were the first to our knowledge. Further, as natriuretic peptide levels (BNP/ NT-proBNP) were included in the entry criteria of BIOSTAT-CHF, comparison of temporal characteristics with

natriuretic peptide levels was also possible in the present study. To note, all patients in BIOSTAT-CHF underwent treatment with furosemide ≥40mg/day or equivalent and received ≤50% of target doses of ACEi/ARBs and beta-blockers at time of enrolment which were then up-titrated in the next three months.

The initial finding of interest was that a low TMAO level (as compared to median) either at baseline or at follow-up was sufficient to confer better outcomes as compared to patients that showed sustained high levels at both initial and follow-up time-points. This was in contrast to natriuretic peptide levels, which showed only better outcomes when there were low levels at follow-up as consistent with previous studies which have shown that lower levels of natriuretic peptide with treatment are associated with favourable outcomes.[16] Another finding of interest was that current guideline-based HF treatment did not affect TMAO levels. In fact, reduction in TMAO levels were not observed in patients with titration of dosages to greater than 50% of the recommended dosage, and a significant increase in TMAO levels was seen in patients achieving less than 50% optimal titration of either ACEi/ARBs or beta-blockers. In contrast, natriuretic peptide levels showed a decrease in response to optimal up-titration (>50%) of ACEi/ARBs and/or beta-blockers as consistent with previous reports that have addressed effects of HF treatment on natriuretic peptide levels.[17,18]

On added value of TMAO levels to risk prediction, TMAO added to risk stratification of the BIOSTAT-CHF risk models (compact and extended) for mortality and mortality/HF at 1, 2 and 3 years, with the exception of the extended model for mortality/HF.

Clinical Implications of all the Available Evidence

Our findings showed that current guideline-based pharmacological treatment in HF impacted natriuretic peptide levels as expected but did not reduce TMAO levels. Studies have shown that dietary modulation (e.g. vegan/vegetarian-based) as well as drugs (e.g. antibiotics,

small compounds) in addition to natural inhibitors (e.g. 3,3-dimethyl-1-butanol, DMB) may lower TMAO levels[19].

Therapeutic intervention to the gut microbiome/dysbiosis and/or intestinal permeability might be potential additive treatments for HF. However, TMAO lowering has yet to be shown to improve outcomes of heart disease and will be a topic for future investigation.

Mechanistic Implications

Although the mechanisms contributing to the increase of TMAO in the setting of HF are likely multifactorial, some plausible mechanisms focused on effects of intestinal dysfunction on increased TMAO include; 1) increased congestion and intestinal dysfunction/congestion in HF leading to dysbiosis of the gut microbiome[20], and 2) congestion leading to increased intestinal permeability[21] resulting in increased entry of the precursor TMA into intestinal blood flow then into the circulation.

Study Limitations

BIOSTAT-CHF was an observational study, therefore optimisation and dosages were decided by clinical discretion. Although there is strong evidence that high blood levels of TMAO correlate with cardiovascular events,[2-7] there remains possibility that increased concentration of TMAO in patients with increased cardiovascular risk may be not a causative relationship. Other potential limitations are that we did not have any information regarding baseline diet, physical activity level and change in weight to adjust for these confounding factors. There is potential for residual measured and/or unmeasured confounding factors to influence association of TMAO with outcomes. Circulating TMAO levels likely depend on a multitude of factors including diet, gut microbiota composition and activity, permeability of the gut-blood barrier, activity of liver enzymes, rate of methylamine excretion, and effects of medications.[22]

CONCLUSION

TMAO levels were able to add to risk stratification of HF in the BIOSTAT-CHF cohort. Elevated levels of circulating TMAO were associated with adverse outcomes (mortality and/or HF hospitalisation) and added to the clinical BIOSTAT-CHF risk models. Serial analysis of TMAO levels with treatment showed that patients with sustained higher levels of TMAO before and after treatment were associated with worse outcomes, and that low levels either at baseline or at follow-up were sufficient for association with better outcomes.

SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Patient characteristics according to median and tertiles of TMAO at visit 1.

Table S2. Differences in characteristics between patients with and without TMAO measurements at visit 1 and visit 2.

Table S3. Cox proportional hazards regression analyses for association of baseline plasma TMAO levels and outcomes in BIOSTAT-CHF model including BMI.

Table S4. Reclassification analysis using continuous reclassification of adding TMAO to BIOSTAT-CHF compact and extended models.

Table S5. Cox proportional hazards regression analyses for association of baseline plasma TMAO levels and outcomes in patients with EF<40% or EF≥40%.

Table S6. Cox proportional hazard regression model including BMI, systolic BP, LVEF and eGFR for the analysis of association with TMAO changes and mortality at 2 years after visit 2.

Table S7. Cox proportional hazard regression for the analysis of association with TMAO changes and mortality at 2 years after visit 2 according to EF less than or greater to 40%.

Table S8. Response of TMAO and BNP levels to guideline-based treatment (less than 50% or not of optimal recommended dosage) according to EF less than or greater to 40%.

Figure S1. Forest plot showing the association with outcome for patients with TMAO levels measured at baseline and secondary visit.

FUNDING

BIOSTAT-CHF was supported by the European Commission [FP7-242209-BIOSTAT-CHF; EudraCT 2010- 020808-29]. The present analysis was supported by the following funding to TS: the Practical Research Project for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus from Japan Agency for Medical Research and Development (AMED) (17ek0210011h0005), the Japan Heart Foundation, the University of Tokyo, the John and Lucille van Geest Foundation and the National Institute for Health Research Leicester Biomedical Research Centre.

CONFLICT OF INTEREST

None of the authors have any disclosures.

REFERENCES

1. Mentz RJ, O'Connor CM. Pathophysiology and clinical evaluation of acute heart failure. Nat Rev Cardiol 2016;13:28-35.

2. Suzuki T, Heaney LM, Bhandari SS, Jones DJL, Ng LL. Trimethylamine N-oxide and prognosis in acute heart failure. Heart 2016;102:841-848.

3. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, Li XS, Levison BS, Hazen SL. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. Circ Res 2015;116:448-55.

4. Tang WH, Wang Z, Shrestha K, Borowski AG, Wu Y, Troughton RW, Klein AL, Hazen SL. Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure. J Card Fail 2015;21:91-96.

5. Schuett K, Kleber ME, Scharnagl H, Lorkowski S, Marz W, Niessner A, Marx N, Meinitzer A. Trimethylamine-N-oxide and Heart Failure With Reduced Versus Preserved Ejection Fraction. J Am Coll Cardiol 2017;70:3202-3204.

6. Troseid M, Ueland T, Hov JR, Svardal A, Gregersen I, Dahl CP, Aakhus S, Gude E, Bjorndal B, Halvorsen B, Karlsen TH, Aukrust P, Gullestad L, Berge RK, Yndestad A. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. Journal of Internal Medicine 2015;277:717-726.

7. Tang WH, Wang Z, Fan Y, Levison B, Hazen JE, Donahue LM, Wu Y, Hazen SL. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: refining the gut hypothesis. J Am Coll Cardiol 2014;64:1908-1914.

8. Romano KA, Vivas EI, Amador-Noguez D, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. MBio 2015;6:e02481.

9. Dolphin CT, Janmohamed A, Smith RL, Shephard EA, Phillips IR. Missense mutation in flavin-containing mono-oxygenase 3 gene, FMO3, underlies fish-odour syndrome. Nat Genet 1997;17:491-494.

10. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, Hazen SL. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576-585.

11. Voors AA, Anker SD, Cleland JG, Dickstein K, Filippatos G, van der Harst P, Hillege HL, Lang CC, ter Maaten JM, Ng L, Ponikowski P, Samani NJ, van Veldhuisen DJ, Zannad F, Zwinderman AH, Metra M. A systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure: rationale, design, and baseline characteristics of BIOSTAT-CHF. European Journal of Heart Failure 2016;18:716-726.

12. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, Falk V, Gonzalez-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GM, Ruilope LM, Ruschitzka F, Rutten FH, van der Meer P, Authors/Task Force M, Document R. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur J Heart Fail 2016;18:891-975.

13. Heaney LM, Jones DJ, Mbasu RJ, Ng LL, Suzuki T. High mass accuracy assay for trimethylamine N-oxide using stable-isotope dilution with liquid chromatography coupled to orthogonal acceleration time of flight mass spectrometry with multiple reaction monitoring. Anal Bioanal Chem 2016;408:797-804.

14. Voors AA, Ouwerkerk W, Zannad F, van Veldhuisen DJ, Samani NJ, Ponikowski P, Ng LL, Metra M, Ter Maaten JM, Lang CC, Hillege HL, van der Harst P, Filippatos G, Dickstein K,

Cleland JG, Anker SD, Zwinderman AH. Development and validation of multivariable models to predict mortality and hospitalization in patients with heart failure. Eur J Heart Fail 2017;19:627-634.

15. Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27:157-172; discussion 207-212.

16. Masson S, Latini R, Anand IS, Barlera S, Angelici L, Vago T, Tognoni G, Cohn JN, Val-He FTI. Prognostic value of changes in N-terminal pro-brain natriuretic peptide in Val-HeFT (Valsartan Heart Failure Trial). J Am Coll Cardiol 2008;52:997-1003.

17. Troughton RW, Frampton CM, Brunner-La Rocca HP, Pfisterer M, Eurlings LW, Erntell H, Persson H, O'Connor CM, Moertl D, Karlstrom P, Dahlstrom U, Gaggin HK, Januzzi JL, Berger R, Richards AM, Pinto YM, Nicholls MG. Effect of B-type natriuretic peptide-guided treatment of chronic heart failure on total mortality and hospitalization: an individual patient meta-analysis. Eur Heart J 2014;35:1559-1567.

18. Thorvaldsen T, Benson L, Dahlstrom U, Edner M, Lund LH. Use of evidence-based therapy and survival in heart failure in Sweden 2003-2012. Eur J Heart Fail 2016;18:503-511.

19. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, Gu X, Huang Y, Zamanian-Daryoush M, Culley MK, DiDonato AJ, Fu X, Hazen JE, Krajcik D, DiDonato JA, Lusis AJ, Hazen SL. Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis. Cell 2015;163:1585-1595.

20. Pasini E, Aquilani R, Testa C, Baiardi P, Angioletti S, Boschi F, Verri M, Dioguardi F. Pathogenic Gut Flora in Patients With Chronic Heart Failure. JACC Heart Fail 2016;4:220-227.

21. Sandek A, Bauditz J, Swidsinski A, Buhner S, Weber-Eibel J, von Haehling S, Schroedl W, Karhausen T, Doehner W, Rauchhaus M, Poole-Wilson P, Volk HD, Lochs H, Anker SD. Altered intestinal function in patients with chronic heart failure. J Am Coll Cardiol 2007;50:1561-1569.

22. Nowinski A, Ufnal M. Trimethylamin N-oxide: A harmful, protective or diagnostic marker in lifestyle disease? Nutrition 2018;46:7-12.

Table 1. Patient characteristics.

	Total cohort (n=2234)	Patients with follow-up visit (n=972)		
		Visit 1	Visit 2	p value
TMAO (*μ*mol/L)	5.9 [3.6-10.8]	5.7 [3.5-9.9]	6.6 [3.7-11.1]	<0.001
Demographics				
Age	70 [61-78]	69 [61-78]		
Male	1654 (74%)	727 (75%)		
Body mass index (kg/m^2)	27.2 [24.2-30.8]	27.2 [24.4-30.9]	27.3 [24.3-30.9]	0.850
Current smoker	312 (14%)	129 (13%)		
Ischemic aetiology	1214 (55%)	524 (54%)		
Hypertension	1401 (63%)	613 (63%)		
Diabetes mellitus	730 (33%)	306 (32%)		
COPD	387 (17%)	172 (18%)		
Previous HF hospitalisation	703 (32%)	292 (30%)		
NYHA class I	52 (2%)	27 (3%)	148 (16%)	<0.001
II	768 (35%)	381 (40%)	572 (60%)	
III	1088 (50%)	451 (48%)	224 (24%)	
IV	260 (12%)	89 (9%)	11 (1%)	
LVEF (%)	30 [25-36]	30 [25-35]	35 [28-42]	<0.001
HFrEF (EF<40%)	1619 (81%)	713 (83%)		
Clinical signs				
Pulmonary congestion	1149 (52%)	463 (49%)	92 (11%)	<0.001
Peripheral oedema	1103 (59%)	372 (52%)	175 (25%)	<0.001
Systolic blood pressure (mmHg)	120 [110-139]	123 [110-140]	122 [110-140]	0.409
Diastolic blood pressure (mmHg)	74 [66-82]	75 [67-85]	75 [65-80]	0.002
Heart rate (beat/min)	76 [67-90]	75 [65-89]	70 [61-80]	<0.001
Medication				
Beta-blocker	1863 (83%)	825 (85%)	906 (93%)	<0.001
ACE inhibitor or ARB	1638 (73%)	720 (74%)	862 (89%)	<0.001
MRA	1193 (53%)	509 (52%)	572 (59%)	<0.001
Diuretics	2232 (100%)	971 (100%)	971 (94%)	<0.001
Laboratory				
Haemoglobin (g/dL)	13.3 [11.9-14.5]	13.4 [12.1-14.6]	13.3 [12.0-14.3]	0.013
BNP (pg/mL)	231 [92-475]	206 [86-408]	132 [51-327]	<0.001
Urea (mmol/L)	11.1 [7.4-17.9]	9.2 [6.7-14.5]	10.2 [7.0-16.0]	<0.001
eGFR* (ml/min/1.73m^2)	62 [48-79]	64 [48-81]	60 [45-78]	<0.001
Sodium (mmol/L)	140 [137-142]	140 [137-142]	139 [137-141]	0.207

Data are presented as median [interquartile range] for continuous variables or n (%) for categorical values. P values are quoted for Wilcoxon matched-pair signed-rank tests for continuous variables and McNemar tests for categorical variables. *Estimated by CKD-EPI formula. ACE=angiotensin-converting enzyme; ARB=angiotensin receptor blocker; BNP=B-type natriuretic peptide; COPD=chronic obstructive pulmonary disease; eGFR=estimated glomerular filtration rate; HF=heart failure; HFrEF=heart failure with reduced ejection fraction; LVEF=left ventricular ejection fraction; MRA=mineralocorticoid receptor antagonist; NYHA=New York Heart Association; TMAO=trimethylamine-N-oxide.

Table 2. Cox proportional hazards regression analyses for association of baseline plasma TMAO levels and outcomes in the BIOSTAT-CHF cohort.

	Unadjusted		**Adding TMAO to Compact model**		**Adding TMAO to Extended model**	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Mortality						
1 year	2.43 (1.93-3.05)	<0.001	1.51 (1.14-2.00)	0.004	1.40 (1.03-1.89)	0.030
2 years	2.29 (1.90-2.77)	<0.001	1.49 (1.18-1.88)	0.001	1.44 (1.12-1.84)	0.004
3 years	2.27 (1.90-2.72)	<0.001	1.47 (1.18-1.84)	0.001	1.42 (1.13-1.80)	0.003
Mortality/HF						
1 year	1.92 (1.61-2.28)	<0.001	1.42 (1.06-1.90)	0.019	1.12 (0.90-1.40)	0.281
2 years	1.91 (1.64-2.22)	<0.001	1.39 (1.09-1.76)	0.007	1.19 (0.98-1.45)	0.077
3 years	1.93 (1.66-2.23)	<0.001	1.37 (1.09-1.72)	0.007	1.21 (1.00-1.46)	0.054

Compact model for all-cause mortality (mortality): age, blood urea (log-transformed), BNP (log-transformed), haemoglobin and use of beta-blocker at baseline. Compact model for mortality or rehospitalisation due to heart failure (mortality/HF): age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, BNP (log-transformed), haemoglobin, sodium and use of beta-blocker at baseline. Extended model for mortality: compact model plus ischemic aetiology, COPD, diastolic blood pressure and sodium. Extended model for mortality/HF: compact model plus current smoker, COPD and eGFR. Data are presented as hazard ratio (HR) and 95% confidence interval (CI). BNP=B-type natriuretic peptide; COPD=chronic obstructive pulmonary disease; eGFR=estimated glomerular filtration rate; HF=heart failure; TMAO=trimethylamine-N-oxide.

Table 3. Response of TMAO and BNP levels to guideline-based treatment (less than 50% or not of optimal recommended dosage).

	TMAO (n=972)			BNP (n=890)		
Dose up-titration	V1 (µmol/L)	V2 (µmol/L)	p value*	V1 (pg/mL)	V2 (pg/mL)	p value*
ACEi/ARBs						
<50%	6.3 [3.8-11.8]	7.1 [3.9-12.9]	0.002	237 [104-469]	172 [70-420]	0.122
≥50%	5.2 [3.4-8.9]	6.2 [3.5-10.3]	0.002	171 [77-342]	108 [39-259]	<0.001
Beta-blocker						
<50%	5.6 [3.5-9.2]	6.7 [3.7-11.2]	<0.001	183 [85-392]	141 [55-369]	0.159
≥50%	5.7 [3.7-10.5]	6.5 [3.7-10.8]	0.084	222 [89-434]	126 [44-277]	<0.001
Both drugs						
Either <50%	5.7 [3.5-9.7]	6.7 [3.7-11.9]	<0.001	203 [86-411]	140 [56-366]	0.053
Both ≥50%	5.6 [3.6-10.1]	6.5 [3.6-10.5]	0.284	208 [88-389]	113 [37-244]	<0.001

p value; initial enrolment visit (V1) to the follow-up visit (V2) were compared using Wilcoxon matched-pair signed-rank test.

ACEi=angiotensin-converting enzyme inhibitor; ARB=angiotensin receptor blocker; BNP=B-type natriuretic peptide; TMAO=trimethylamine N-oxide.

FIGURE LEGENDS

Figure 1. Cumulative incidence of events across TMAO tertiles.

A: Cumulative incidence plot for all-cause mortality (mortality) at 3 years stratified by TMAO tertiles. B: Cumulative incidence plot of mortality and/or rehospitalisation due to heart failure (mortality/HF) at 3 years stratified by TMAO tertiles. Number of events are shown below.

Figure 2. Forest plot showing the association with outcome for patients with TMAO (top) and BNP (bottom) levels measured at baseline and secondary visit.

Patients were divided into four groups according to TMAO (top) and BNP (bottom) concentrations at the initial enrolment visit (V1) and follow-up visit (V2) relative to the median of each visit point (TMAO; 5.7 µmol/L and 6.6 µmol/L, BNP; 206 pg/mL and 132 pg/mL, for V1 and V2 repsectively). H/H=high V1 and high V2; H/L=high V1 and low V2; L/H=low V1 and high V2; L/L=low V1 and low V2. Cox proportional hazards regression modelling was used to compare the risk of mortality at 2 years after V2 among the four groups of patients using L/L as the reference on each occasion (A and C unadjusted, B and D adjusted with renal function (eGFR at V1) for TMAO and BNP respectively). Data are presented as hazard ratio (HR) and 95% confidence interval (CI). BNP=B-type natriuretic peptide; eGFR=estimated glomerular filtration rate; TMAO=trimethylamine-N-oxide.

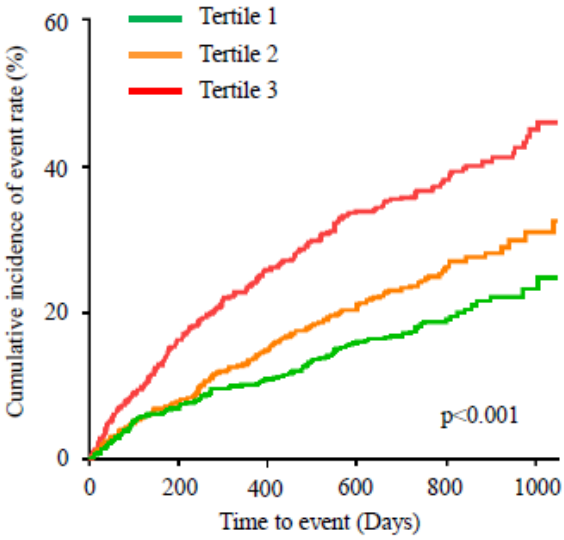
Figure 3. Classification tree to show risk stratification for mortality at 2 years using combined measurements at baseline and secondary visit for TMAO (top) and BNP (bottom).

A: Classification tree using plasma TMAO level at the initial enrolment visit (V1) as the initial classifier, followed by plasma TMAO level at follow-up visit (V2) enables effective selection of low- and high-risk groups of patients (main body) and increased cumulative event risk in Group C compared to Groups A and B (inset). B: Classification tree using plasma BNP level at V1 as the

initial classifier, followed by plasma BNP level at V2 enables effective selection of low- and high-risk groups of patients (main body) and increased cumulative event risk in Groups B and D compared to A and C (inset). Data are presented as hazard ratio (HR) and 95% confidence interval (CI). Number of events are shown below. BNP=brain natriuretic peptide; TMAO=trimethylamine-N-oxide.

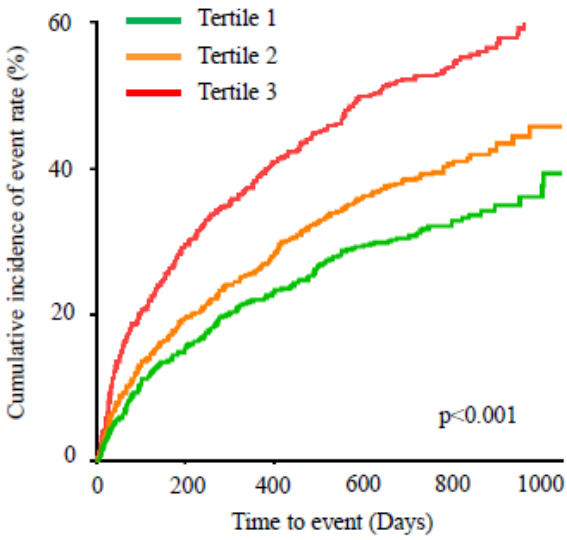
Figure 1

A



Tertile 1	744	687	648	453	222	50
Tertile 2	745	680	619	449	205	55
Tertile 3	745	616	536	356	199	57

B



Tertile 1	744	625	557	383	185	39
Tertile 2	745	592	521	358	162	32
Tertile 3	745	519	427	262	147	36

Figure 2

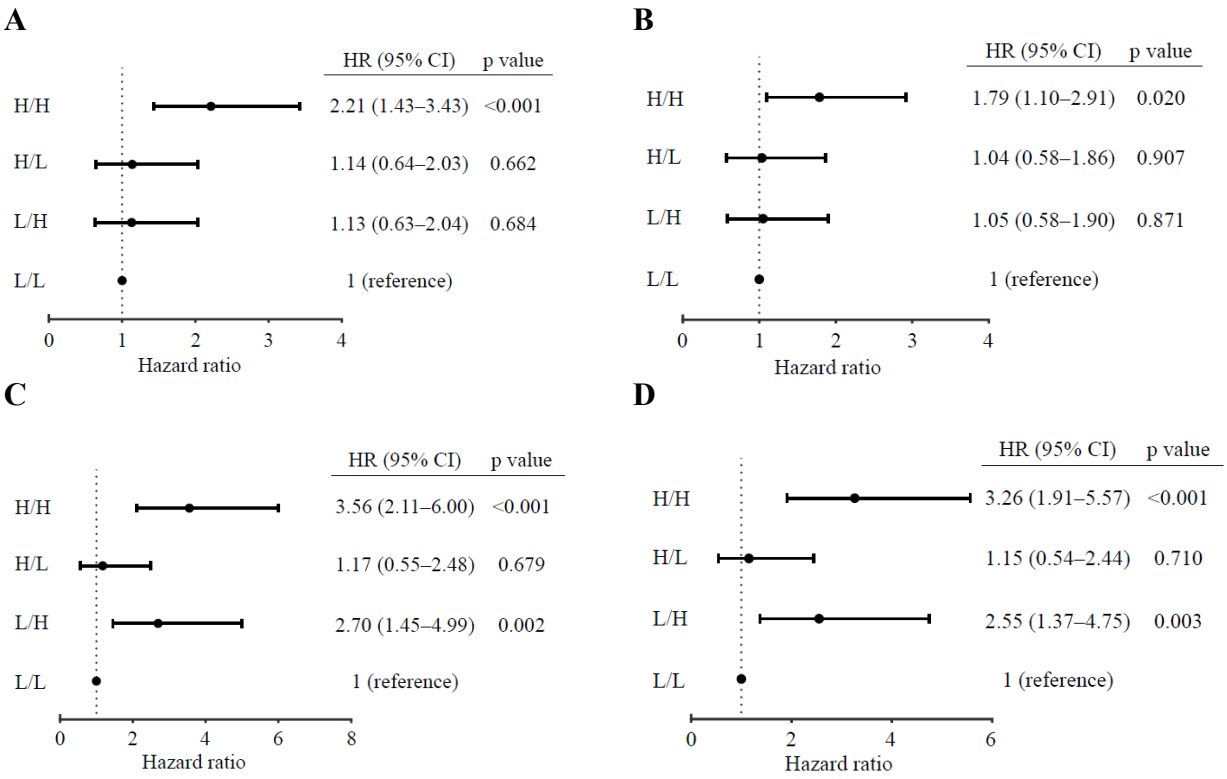
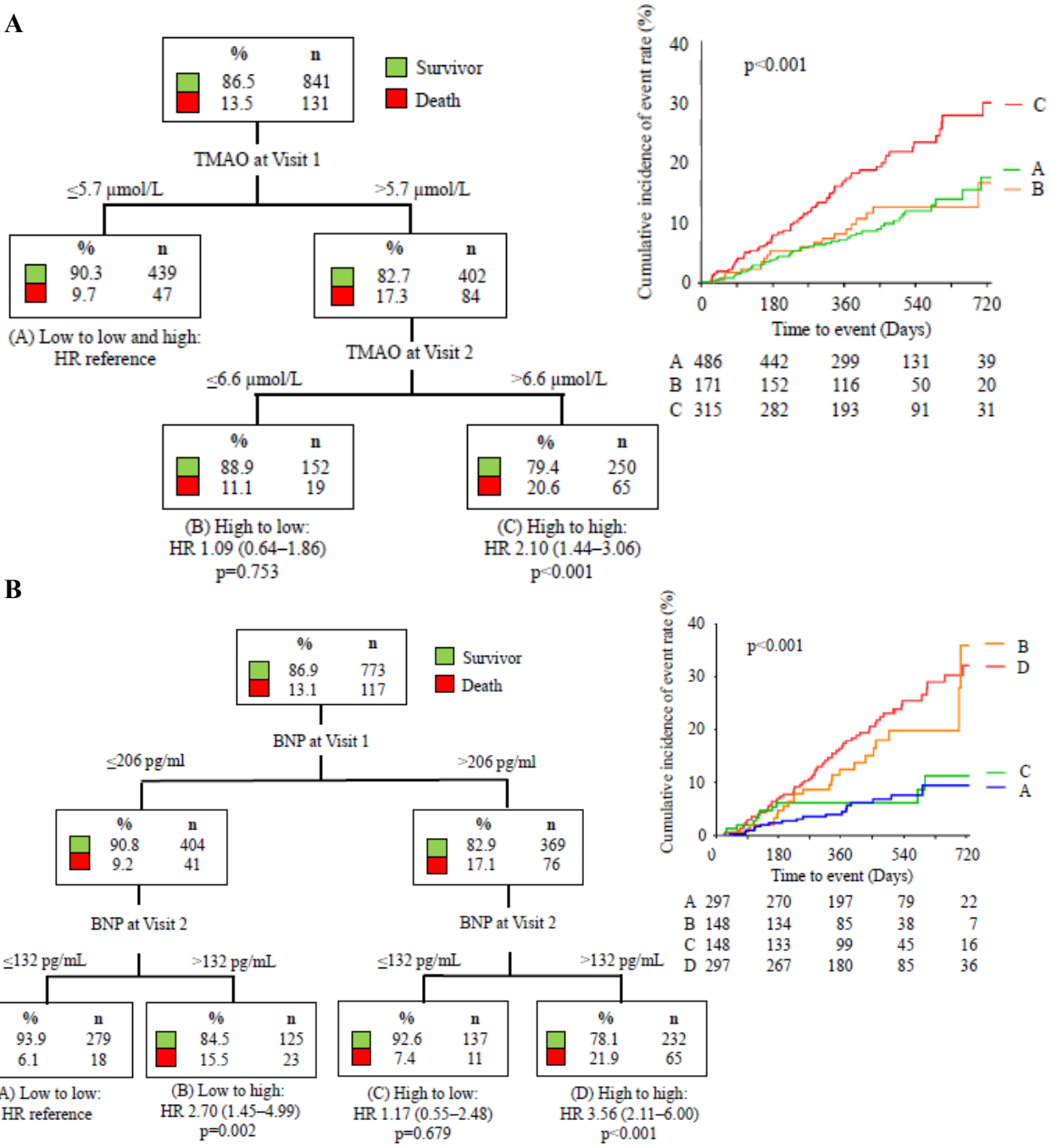


Figure 3


A
% n
86.5 841
13.5 131
Survivor
Death
TMAO at Visit 1
≤5.7 µmol/L
>5.7 µmol/L
% n
90.3 439
9.7 47
% n
82.7 402
17.3 84
(A) Low to low and high:
HR reference
TMAO at Visit 2
≤6.6 µmol/L
>6.6 µmol/L
% n
88.9 152
11.1 19
% n
79.4 250
20.6 65
(B) High to low:
HR 1.09 (0.64–1.86)
p=0.753
(C) High to high:
HR 2.10 (1.44–3.06)
p<0.001
Cumulative incidence of event rate (%)
p<0.001
Time to event (Days)
A 486 442 299 131 39
B 171 152 116 50 20
C 315 282 193 91 31
B
% n
86.9 773
13.1 117
Survivor
Death
BNP at Visit 1
≤206 pg/ml
>206 pg/ml
% n
90.8 404
9.2 41
% n
82.9 369
17.1 76
BNP at Visit 2
BNP at Visit 2
≤132 pg/mL
>132 pg/mL
≤132 pg/mL
>132 pg/mL
% n
93.9 279
6.1 18
% n
84.5 125
15.5 23
% n
92.6 137
7.4 11
% n
78.1 232
21.9 65
(A) Low to low:
HR reference
(B) Low to high:
HR 2.70 (1.45–4.99)
p=0.002
(C) High to low:
HR 1.17 (0.55–2.48)
p=0.679
(D) High to high:
HR 3.56 (2.11–6.00)
p<0.001
Cumulative incidence of event rate (%)
p<0.001
Time to event (Days)
A 297 270 197 79 22
B 148 134 85 38 7
C 148 133 99 45 16
D 297 267 180 85 36

Supplementary Material